K.,M.S.K. (2001) Identification of the hemoglobin scavenger receptor, *Nature 409*, 198-201) as template. The PCR generated DNA fragment was ligated into the internal Pst I site (position 3056-3061) and the Not cloning site of the previously described full-length HbSR pcDNA(+) plasmid (Kristiansen,M., Graversen,J.H., Jacobsen,C., Sonne,O., Hoffman,H., Law,A.S.K., and K.,M.S.K. (2001) Identification of the hemoglobin scavenger receptor, *Nature 409*, 198-201). This procedure substituted bases 3136 to 3351, encoding the transmembrane region and the cytoplasmatic tail of HbSR, with a stop codon. The expression product from the transfected CHO cells was as expected secreted into the medium as a soluble protein. Minor amounts were purified from the medium by haptoglobin-hemoglobin affinity chromatography as described previously (Kristiansen,M., Graversen,J.H., Jacobsen,C., Sonne,O., Hoffman,H., Law,A.S.K., and K.,M.S.K. (2001) Identification of the hemoglobin scavenger receptor, *Nature 409*, 198-201).

Expression of recombinant fragments of HbSR corresponding to SRCR 1-6 and SRCR 5-9

cDNA encoding SRCR domain 1-6 and SRCR domain 5-9 extended with Hind III and Xho I restriction sites were amplified by polymerase chain reactions (PCR) using full-length HbSR cDNA (Kristiansen,M., Graversen,J.H., Jacobsen,C., Sonne,O., Hoffman,H., Law,A.S.K., and K.,M.S.K. (2001) Identification of the hemoglobin scavenger receptor, *Nature 409*, 198-201) as template. The PCR products were subcloned into the expression vector pSecTag2B (Invitrogen, Groningen, The Netherlands) by use of the restriction sites HindIII and XhoI. Plasmids were transformed into *E. coli* DH5α cells (Clontech, Palo Alto, CA, USA), and plasmid DNA isolated and sequenced prior to transfection. The following primers were used for construction of the fragments: SRCR domain 1-6: forward 5'-caagcttggaacagacaaggagctg-3' and reverse 5'-cctcgagtcctgagcagattacagag-3'. SRCR domain 5-9: forward 5'-caagcttcacagggaacccagactg-3' and reverse 5'-cctcgagatctgtgcaattcactgc-3'.

CHO-K1 cells were transfected with plasmids and expression products detected by Western blotting using a rabbit polyclonal antibody against human HbSR, as described (Kristiansen,M., Graversen,J.H., Jacobsen,C., Sonne,O., Hoffman,H., Law,A.S.K., and K.,M.S.K. (2001) Identification of the hemoglobin scavenger receptor, *Nature 409*, 198-201). Recombinant HbSR SRCR 1-6 was purified by Hp-Hb-affinty chromatography as described for full length recombinant HbSR, while HbSR SRCR domain 5-9 failed to bind to Hp-Hb-Sepharose. Binding of Hp-Hb to the HbSR derivative corresponding to SRCR domain 1-6 immobilized on a BIACore CM5 chip was confirmed by BIACore binding analysis (Biacore International AB, Uppsala, Sweden) as described (Kristiansen,M., Graversen,J.H., Jacobsen,C., Sonne,O., Hoffman,H., Law,A.S.K., and K.,M.S.K. (2001) Identification of the hemoglobin scavenger receptor, *Nature 409*, 198-201). For the sensorgram shown on figure 10 the density of HbSR and HbSR SRCR domain 1-6 coupled on the chip was 0.0659 and

0.0370 pmol/mm^2, respectively, the concentration of Hp(1-1)-Hb used was 280 nM or 0.04 mg/ml, and the buffer used was CaHBS from BIACore.

Purification and characterization of an autoproteolytic HbSR fragment

In the process of purifying HbSR an autoproteolytic product of HbSR co-purified on Hp-Hb-sepharose. N-terminal sequencing of the fragment revealed the following sequence for the major form: DGVTE, corresponding to amino acid residues 265-269 of HbSR. Estimated by the mobility in SDS-PAGE analysis the fragment correspond to HbSR amino acid residues 265-1116, thus all of HbSR except SRCR domain 1 and 2.

Conclusion

Fragments of HbSR containing SRCR domains 1-6 and 3-9 bound Hp-Hb, while a fragment containing HbSR domain 5-9 failed to bind Hp-Hb. Thus SRCR domain 3 and 4 are necessary for HbSR binding to Hp-Hb.

Example 8

Production of antibodies directed to Hp-Hb complex and CD163 receptor.

Two Fab antibody libraries expressed on phage to isolate Fab antibodies for structure-function analysis on the Hp-Hb complex-CD163 interaction.

Proteins and chemicals- Human CD163 was purified as described (Kristiansen, M., Graversen, J. H., Jacobsen, C., Sonne, O., Hoffman, H. J., Law, S. K., and Moestrup, S. K. (2001) Nature 409(6817), 198-201.). Hb and Hp (mixed phenotypes, 1:1 or 2:2 forms) purchased from Sigma, were mixed on ice in equal molar amounts to allow for complex formation and dialyzed against HEPES-containing buffer at pH 7.4 before use. Anti-Hb and anti-Hp antibodies were purchased from Sigma. An anti-M13-peroxidase coupled antibody and mixed deoxy-nucleotides were purchased from Amersham-Pharmacia Biotech. DNA modifying enzymes were purchased from Invitrogen and New England Biolabs. Oligonucleotides were obtained from DNAtechnology, Taq polymerase was from Promega. Proteins were labeled using the chloramine-T method. All other reagents and chemicals were reagent grade (Sigma and Merck).

Construction of phage-displayed Fab libraries- Phage display libraries were constructed using the pCOMB3X system (Andris-Widhopf, J., Rader, C., Steinberger, P., Fuller, R., and Barbas, C. F., 3rd. (2000) J Immunol Methods 242(1-2), 159-81.). The pCOMB3X phagemid which was kindly supplied by Dr. C.F. Barbas (the Scripps Research Institute in La Jolla,

USA). Two Balb/C mice were immunized three times with 10 µg purified Hp-Hb complexes diluted in incomplete Freund's adjuvans during a period of 6 weeks. Subsequently, mice were sacrificed and spleens were isolated. Using a filter, single cell suspensions were obtained which were suspended in Trizol reagent (Invitrogen, the Netherlands) and RNA was isolated following the instructions of the supplier. Using approximately 10 µg total RNA, first strand synthesis was carried out using the SuperScript II first strand synthesis system (Invitrogen, the Netherlands) and 3' end primers specific for the mouse first constant domain of the heavy chain or for the mouse kappa light chain constant domain (Kang, A. S., Burton, D. R., and Lerner, R. A. (1991) Methods: A Companion to Methods in Enzymology 2(2), 111-118) exactly following the procedure from the supplier. In an extensive set of polymerase chain reactions using well-described primers (Kang, A. S., Burton, D. R., and Lerner, R. A. (1991) Methods: A Companion to Methods in Enzymology 2(2), 111-118), specific cDNA's encoding variable and first constant domains of the IgG1 and IgG2a heavy chains and complete IgG1 and IgG2a kappa light chains were amplified. Optimal temperature conditions were sorted out using a Stratagene Robocycler. Amplified products were subsequently purified, digested and ligated into the restriction sites of cleaved pCOMB3X as described in (Kang, A. S., Burton, D. R., and Lerner, R. A. (1991) Methods: A Companion to Methods in Enzymology 2(2), 111-118). Electrocompetent *Escherichia coli* XL1-Blue cells (Stratagene) were transformed using an Eppendorf electroporator and ligation efficiency and size of the library determined. Upon infection with VCS M13 helper phage (Stratagene) phage-antibody libraries were obtained that on average consisted of $5x10^5$ individual colonies.

Selections of anti-Hb-Hp and anti-CD163 antibody phage- Phage selections were performed in 96-well plates (NUNC, Denmark) coated with 1 µg of purified Hp-Hb complexes or CD163 and blocked with BSA. Pannings were done essentially as described (Horn, I. R., Moestrup, S. K., van den Berg, B. M., Pannekoek, H., Nielsen, M. S., and van Zonneveld, A. J. (1995) J Biol Chem 270(20), 11770-5.). During the biopanning phage were eluted using glycine-adjusted 50 mM hydrochloric acid, pH 2.1. Selection rounds were repeated another 3 times and the output/input ratio was calculated after titration of phage. These ratios indicate the phage enrichment values during the procedure. In Fig. 11 the output/input ratios per selection round are shown as well as the results of a phage ELISA. As can be seen in the figure, in both selections a strong enrichment for binding Fab phage has occurred, mounting to approximately 100-fold for the Hp-Hb complex-selection and to 1000-fold for the anti-CD163 selection. Upon testing randomly picked clones from the four consequetive rounds of selections, we found binding clones in the third round of selection for both antigens. The results of two ELISA assays are shown in Fig. 11, panels *B* and *D*. In total, a hundred clones were screened from the second and third round of selection. Postive clones were not further enriched in the fourth round of selection. To investigate if selected clones were different, PCR fingerprinting with different restriction enzymes were performed on all positive clones. The